

SUGAR RELEASE AND PENETRATION IN INSECT FAT BODY :
RELATIONS TO REGULATION OF HAEMOLYMPH
TREHALOSE IN DEVELOPING STAGES OF
HYALOPHORA CECROPIA

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In insects, the fat body is the chief site of synthesis of the disaccharide trehalose, which is generally the predominant circulating sugar (review: Wyatt, 1967). There is evidence for homeostatic regulation of haemolymph trehalose levels (Saito, 1963; Friedman, 1967; Wyatt, 1967; Nettles, Parro, Sharbaugh and Mangum, 1971) and, to account for this, feedback mechanisms have been proposed. While inhibition and activation of enzymes concerned with trehalose synthesis have been demonstrated *in vitro* (Murphy and Wyatt, 1965; Friedman, 1968), the mechanisms responsible for regulation *in vivo* are unclear. One hitherto unanswered question is how an insect can maintain its haemolymph sugar at distinctly different levels in its different developmental stages, as, for example, the cecropia silkmoth has been shown to do (Wyatt, 1967).

The dynamic relations between intracellular and extracellular trehalose are clearly important for the regulation of trehalose synthesis, yet very little is known about these. Mochmacka and Petryszyn (1959) found trehalose to be higher in haemolymph than in "bled tissues" of pupae of the sphingid *Clerio euphorbiae*, and larval and adult tissues of this species apparently contained no trehalose. Fat body of cecropia silkmoth larvae (Wyatt, 1967, page 297) and wax moth larvae (Lenartowicz, Zaluska and Niemierko, 1967), after rinsing in saline solutions, was found to contain trehalose at levels much lower than those in the haemolymph of the respective insects. These reports suggest that movement of trehalose from fat body to haemolymph might require active transport, albeit active transport of sugars has never been demonstrated in an insect. In fact, the absorption of sugars from the insect gut, in contrast to analogous processes in vertebrates, depends upon simple diffusion (Wyatt, 1967).

Fat body of larval blowflies (*Phormia regina*), on the other hand, contained trehalose even though none could be found in the haemolymph of the same stage (Wimer, 1969).

In view of these problems, we have examined the relations between fat body and haemolymph sugar in several insect species. We find that fat body analyzed without prior rinsing may contain sugar at levels lower than, equal to, or higher than those in the haemolymph, but during rinsing in isosmotic media much of the tissue sugar is rapidly released. During ontogeny of the cecropia silkmoth, the quantitative relationships between haemolymph and fat body trehalose change markedly. Correlated with these changes are alterations in fat body function with

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respect to release of endogenous and penetration of exogenous trehalose, which we interpret as reflecting changes in the cell membrane having regulatory significance.

MATERIALS AND METHODS

Animals and media

Most of the experiments were conducted with cecropia silkmoths (*Hyalophora cecropia*), reared from genetically mixed stock either outdoors on wild cherry foliage or in the laboratory at 25° C on an artificial diet (Riddiford, 1968) under 16 hours light per day. After spinning their cocoons, outdoor reared animals were held at 25° C for 2–4 months and then (a) chilled at 6° C to activate the neuroendocrine system for development, or (b) debrained to establish permanent diapause (Williams, 1946) and then kept at 15° C, or (c) left at 25° C, at which temperature diapause persisted in most individuals for several months. In many instances, pupal respiration was measured, and the criterion for diapause was an O₂ consumption of less than 20 µl per gram live weight per hour (Schneiderman and Williams, 1953). Pharate adults ("developing adults") were obtained by incubating previously chilled pupae at 25° C, and stages were recognized as described by Schneiderman and Williams (1954). Insects of other species, used in a few experiments, were obtained as described in the footnotes to Table I.

Injections were made through the thoracic tergum of pupae in volumes not more than 50 µl, and between the first pair of dorsal tubercles of larvae in not more than 100 µl. Experimental injury consisted of making 20 punctures with a 25 gauge needle in the thoracic tergum, which were then sealed with melted paraffin wax. Haemolymph was collected at the time of injury by puncturing a wing sac with the tip of a scalpel and then sealing the hole. When haemolymph was collected for use in media, a few crystals of phenylthiourea were added to prevent darkening due to the action of tyrosinase.

The media used for rinsing and incubation of tissue from all insects except *Blaberus discoidalis* were: (i) that of Reddy and Wyatt (1967) (RW medium) containing NaCl 20 mM, KCl 80 mM, CaCl₂ 4 mM, MgCl₂ 15 mM, phosphate 8 mM, and 20 amino acids (total 91 mM), the sugars in the published medium being omitted, with pH 6.5 and osmotic pressure approximately 450 milliosmolar; (ii) modified RW medium in which the concentrations of NaCl and KCl were reduced to 4 and 50 mM, respectively (total 360 milliosmolar); and (iii) high K⁺-Mg⁺⁺ lepidopteran saline, modified after Weevers (1966) and Pan, Bell and Telfer (1969), which contained NaCl 4 mM, KCl 140 mM, CaCl₂ 4 mM and MgCl₂ 15 mM (total 350 milliosmolar). In initial experiments RW medium was used, while later the modified RW medium was substituted, and no differences were observed in the results. For *B. discoidalis*, a medium was used consisting of NaCl 130 mM, KCl 10.3 mM, CaCl₂ 4.5 mM, MgCl₂ 8 mM, and phosphate buffer 5 mM, together with the amino acid mixture of RW medium (pH 6.5, 425 milliosmolar; cf. Van Asperen and Van Esch, 1956).

The sugar content of haemolymph and fat body, and efflux from fat body in vitro

Haemolymph and fat body were taken from individual insects for analysis. The fat body was dissected out, blotted by drawing it repeatedly across Glassine

weighing paper (Eli Lilly and Company, Indianapolis, Indiana), and portions of 50–500 mg were homogenized for analysis immediately, or after immediate freezing, or after incubation at 25° in not less than 1.8 ml of medium. For analysis, blotted tissue samples were weighed to the nearest milligram on a torsion balance and ground in glass homogenizers in three volumes of 6.7% trichloroacetic acid. Homogenates were transferred to 0.5 ml capped polyethylene tubes, and centrifuged at $8000 \times g$ in a Microfuge (Model 152; Beckman Instruments, Palo Alto, California). Ten-microliter samples of the clear supernatant were transferred into 0.29 ml of 95% ethanol to precipitate the glycogen, and recentrifuged. Samples (150 microliters) for determination of trehalose were then taken and evaporated to dryness below 60° C in a vacuum oven, and 1.20 ml of fresh anthrone reagent (Mokrasch, 1954), with increased water to allow for the use of dry samples, was added to each. The tubes were heated in a boiling water bath for 5 minutes, then cooled in ice and absorbance was determined at 620 nm with a Zeiss spectrophotometer. Haemolymph trehalose was measured by treating 1–10 microliters of whole haemolymph (which contains negligible ethanol-precipitable carbohydrate) with anthrone reagent as just described. Glucose standards were run simultaneously.

The sugar measured by the anthrone reaction is designated as trehalose. In haemolymph of the cecropia silkworm, trehalose has been identified by specific methods and only minute amounts of other sugars are present (Wyatt and Kalf, 1957; Wyatt, 1967). We tested an extract of fat body from diapausing cecropia pupae for reactivity with glucose oxidase with and without prior treatment with trehalase (prepared from *Tenebrio molitor* larvae) and found abundant trehalose but no detectable free glucose. The assumption that trehalose is the significant sugar in our experiments with *H. cecropia* therefore seems justified. In the other insect species, we have not established what proportion of the anthrone-reactive sugar is trehalose. In the results that follow, data obtained by the anthrone reaction are expressed in the Figures and Tables as glucose equivalents, while in the text, sugar is assumed to be trehalose and is expressed on a molar or molal basis as such (= glucose equivalents \times 0.5).

The amount of adhering haemolymph in a number of fat body tissue samples from cecropia pupae was determined by marking the haemolymph with ^{14}C -inulin, injected 3 hours before the animals were dissected, as described by Cherbas and Cherbas (1970). The content of haemolymph in blotted fat body was found to be 20–40%, the majority of samples having 30–35%. In most experiments, haemolymph contamination was not measured, but was assumed to be 30%; thus, cellular volume was estimated as blotted tissue weight \times 0.7, the specific gravity being taken as 1.0. Underestimating haemolymph contamination in the tissue samples would minimize the true differences between trehalose concentrations in the cells and in the haemolymph.

Concentrations of sugar in haemolymph can be expressed in molarity, based on volume, but to permit osmotic comparison with the intracellular fluid it was desirable to express them also in molality. For this purpose, 100 ml of haemolymph was assumed to contain 85 g of water, a mean value appropriate for cecropia pupal haemolymph, though for larval blood the true water content is probably somewhat higher (Wyatt and Kalf, 1957). The water content of fat body cells was

estimated by weighing portions of the tissue before and after exhaustive drying, correction being made for extracellular fluid with the aid of radioactive inulin. Based on several measurements on tissue from fully-grown feeding larvae and from diapausing pupae of *ceropia*, the following percentages of cellular fresh weight were assumed for intracellular water: larval fat body, 50%; pupal fat body, 70%. With other insect species, the value of 50% was used for fat body from the larval or adult stages, and 70% for pupal tissue.

Treatment of data

For a given treatment, the concentration of trehalose in the fat body was frequently found to bear a constant relationship to that in the haemolymph, while the absolute levels varied considerably among individuals of a group. Consequently, the data were normalized by multiplying all individual values for fat body trehalose by the ratio: group mean concentration in haemolymph/individual concentration in haemolymph. This method is comparable to expressing individual concentrations as percentages of haemolymph trehalose, but also permits comparison of the levels of sugar in different batches of animals at the same or different stages of development.

The sexes of the animals used were noted, but no differences related to sex were observed in haemolymph trehalose, fat body trehalose, or the effect of any treatment.

Uptake of ^{14}C -solute

Uptake of $1\text{-}^{14}\text{C}$ -glucose, $\text{U-}^{14}\text{C}$ -glycerol and $6\text{-}^{14}\text{C}$ -trehalose into fat body *in vitro* was measured with the aid of ^3H -inulin as a marker for extracellular fluid. Ten microliters of a stock solution containing 2×10^4 counts/min per microliter each of ^{14}C -solute and ^3H -inulin were added to 1.00 ml of RW medium in a 10 ml beaker. A $10 \mu\text{l}$ sample of this mixture was taken for determination of the initial $^{14}\text{C}/^3\text{H}$ ratio. Fat body (0.2–1.0 g, pooled from 2–5 animals) was added, continuous shaking was commenced, and $5 \mu\text{l}$ samples of the medium were taken approximately each minute for 10 minutes. Difficulty was experienced from clogging of the micropipets by fragments of tissue, and toward the end of this work a vessel was constructed containing a suspended well bottomed with $100\text{-}\mu\text{m}$ nylon mesh which provided a pool of tissue-free medium. This allowed more frequent and accurate sampling. The samples were mixed with 0.1–0.2 ml of NCS (Amersham-Searle, Des Plaines, Illinois) to which 5 or 10 ml of toluene phosphor scintillation fluid was then added. Radioactivity was counted in a Packard scintillation counter.

Because of the difficulty in obtaining precisely measured samples of medium, calculations were based on isotope ratios, which are independent of sample size. The initial ratio of ^{14}C -solute/ ^3H -inulin (R_0) represented no penetration of solute, while a calculated theoretical final ratio (R_x) represented equal distribution of ^{14}C -solute in fat body intracellular water and the incubation medium (see Equation 1). From the observed ratio at any time (R_t), the percentage penetration could be calculated (Equation 2). The $^{14}\text{C}/^3\text{H}$ ratio generally became essentially constant within 10 minutes of incubation, and in the case of diapausing

pupal fat body did not change appreciably during an additional 50 minutes of incubation.

$$R_{\infty} = \frac{m + hs}{m + hs + (1-h)ds} \times R_0 \quad (1)$$

Inserting values of 0.3 for h and 0.5 or 0.7, respectively, for d ,

$$R_{\infty} = \frac{m + 0.3s}{m + 0.65 \text{ (or } 0.79)s} \times R_0 \quad (1a)$$

$$\text{Percentage of penetration of solute} = \frac{R_0 - R_t}{R_0 - R_{\infty}} \times 100 \quad (2)$$

Where m = volume of incubation medium (1.0 ml), s = volume of tissue sample, assuming 1 g = 1 ml, h = fraction of haemolymph in tissue sample (generally assumed to be 0.3), d = fraction of water in fat body cells (assumed to be 0.5 for larval tissue or 0.7 for pupal tissue), R_0 = initial ratio of $^{14}\text{C}/^3\text{H}$ in the medium, R_{∞} = theoretical final ratio of $^{14}\text{C}/^3\text{H}$, assuming complete penetration of cell water by ^{14}C -solute and no penetration by ^3H -inulin, and R_t = observed ratio of $^{14}\text{C}/^3\text{H}$ after incubation.

RESULTS

The sugar content of fat body and haemolymph of insects at different stages of development

The levels of anthrone-reactive sugar, which in cecropia silkmoths will be regarded as trehalose (see Materials and Methods) were measured in unrinsed fat body (corrected for measured or estimated adhering haemolymph) from several developmental stages of four insect species belonging to three orders (Table I). The relationships between sugar in haemolymph and in fat body were fairly constant among individuals of the same species and stage of development, but showed substantial variation in different species and stages. Thus, fat body sugar was significantly higher than haemolymph sugar in adults of *Blaberus*, about equal to haemolymph sugar in *Phyllophaga* larvae, and lower than haemolymph sugar in *Manduca* larvae and pupae. In *Hyalophora cercopia*, as previously observed (Bade and Wyatt, 1962; Wyatt, 1967), the level of trehalose in the haemolymph changed markedly in different developmental stages, being 40–50 mM in the fully-grown larva, falling to 8–15 mM in the diapausing pupa, and rising again to 30–40 mM in the pharate adult. Intracellular sugar underwent less extreme change, and was found in the fat body of larvae and early pharate adults at nearly their respective haemolymph levels and in that of pupae at more than twice their haemolymph level. Comparable changes between developmental stages were not observed in *Manduca sexta*.

When fat body from the cockroach *B. discoidalis* or larvae, pupae or pharate adults of the cecropia silkmoth was incubated briefly in culture media (see Materials and Methods), a significant proportion of its sugar content escaped (Tables I, II). Since this phenomenon appeared to represent a potential source of serious misconception arising from analysis of rinsed tissues, and further might have physiological importance, we decided to investigate it further.

TABLE 1

Sugar concentrations in haemolymph and fat body from various insects. Portions of fat body were incubated for 10 minutes in RII[†] medium (or Blaberus medium) with or without 2% bovine serum albumin (BSA). Sugar was determined by anthrone, and concentrations are expressed in molalities as glucose equivalents in haemolymph or fat body water \pm S.E.M. The results for fat body were corrected for adhering haemolymph or medium, and the water content of haemolymph and fat body was estimated, as described in Materials and Methods

Order	Dictyoptera	Coleoptera	Lepidoptera			
Species	<i>Blaberus discoidalis</i> *	<i>Phyllophaga</i> species***	<i>Manduca sexta</i> ***		<i>Hyalophora cecropia</i>	
Stage	Adults	Larvae	Larvae	Diapause pupae	Larvae†	Larvae††
Number of animals	4	3	4	3	2	4
Sugar concentrations (as glucose)						
Haemolymph (millimolar)	49.1 \pm 4.9	68.2 \pm 3.6	71.0 \pm 8.9	80.7 \pm 5.3	86.7 \pm 34.0	59.6 \pm 3.8
Haemolymph (millimolar)	57.8 \pm 5.8	80.2 \pm 4.2	83.5 \pm 10.5	94.9 \pm 6.2	102.1 \pm 40.0	70.1 \pm 4.5
Fat body, unrinsed (millimolar)	107.2 \pm 16.2	86.9 \pm 3.1	46.6 \pm 5.4	57.1 \pm 6.2	110.3 \pm 11.8	84.7 \pm 6.7
Fat body, incubated (millimolar)						
Protein-free medium	71.8 \pm 5.7				46.7 \pm 8.0	44.2 \pm 0.9
Medium + 2% BSA	80.2 \pm 7.3				54.7 \pm 8.3	55.4 \pm 6.7
Sugar molality ratio, unrinsed fat body/haemolymph	1.85	1.08	0.56	0.60	1.08	1.21
Retention in fat body in medium without protein (%)	67				42	52
Retention in fat body in medium with protein (%)	75				50	65

* Reared at 25° C on corn meal and Purina rat chow.

** Fully grown larvae collected from leaf mold in early May and stored for 2 weeks at 15° C before analysis.

*** Reared at 25° C on artificial diet (Yamamoto, 1968) on a light regime of 12L/12D. The larvae were taken on the day of burrowing and the pupae 2 months after pupation.

† Laboratory reared as described in Methods, taken 1 day before spinning.

†† Outdoor reared, acclimatized at 25° C, 0–4 days before spinning.

‡ Outdoor reared, held for 3 months at 25° C after spinning.

‡‡ Outdoor reared, chilled for 4 months at 6° C, then returned to 25° C for development.

TABLE II

Concentrations of trehalose in haemolymph and fat body of various batches of *cecropia* pupae, and the effects of different media on retention of trehalose in fat body. Batches I-III were reared out of doors on foliage and were collected as fresh cocoons on different dates: batch I, June 22, 1970, batch II, July 19, batch III, August 26. Batch IV was reared in the laboratory on artificial diet and spun cocoons in February, 1971. When respiration was measured, only pupae using less than 20 μ l O₂ per gram-hour (characteristic of diapause) were used. The haemolymph content of fat body samples was measured (see Methods) in Group A, and was assumed to be 30% in all other groups. Other particulars were as in Table I

	Unchilled diapause pupae							
	A	B	C	D	E	F	G	H
Batch number, and age of pupae when used (months)	I (4)	I (4)	II (4)	III, IV (3-4)	III (4.5)	III (8)	IV (3)	II (7)
Number of experiments, ** and total number of animals	2, 7	2, 6	2, 7	3, 8	2, 6	2, 4	1, 3	1, 3
Respiration measured	No	No	Yes	Yes	Yes	Yes	No	Yes
Sugar concentrations	19.0 \pm 2.4	14.8 \pm 2.1	13.2 \pm 0.8	32.3 \pm 3.5	27.6 \pm 1.9	33.1 \pm 4.2	42.6 \pm 2.0	39.1 \pm 3.0
Haemolymph (millimolar)	22.4 \pm 2.8	17.4 \pm 2.5	15.5 \pm 0.9	38.0 \pm 4.1	32.5 \pm 2.2	38.9 \pm 4.9	50.1 \pm 2.4	46.0 \pm 3.5
Haemolymph (millimolar)	42.4 \pm 2.1	40.4 \pm 2.6	38.0 \pm 2.8	40.7 \pm 3.2	36.8 \pm 3.3	28.8 \pm 3.1	37.2 \pm 2.7	55.0 \pm 2.5
Fat body, unrisen (millimolar)								
Fat body, incubated (millimolar)	17.3 \pm 2.4	16.3 \pm 2.4	19.0 \pm 0.8	22.3 \pm 1.7(5)***	20.2 \pm 0.9	19.6 \pm 2.9	31.6 \pm 2.4	29.8 \pm 5.3
RW medium								
RW + 2% haemolymph protein								
RW + 2% BSA			33.9 \pm 3.8	36.4 \pm 2.4	23.8 \pm 1.0	25.1 \pm 3.6		
RW + 2% PVP				34.0 \pm 6.1(3)***	19.9 \pm 1.9	23.4 \pm 3.8		
K-Mg ringet + 2% haemolymph protein							17.3 \pm 1.7	
K-Mg ringer + 2% BSA								
Sugar molality ratio, fat body/haemolymph	1.89	2.32	2.45	1.07	1.13	0.74	0.74	1.20
Retention in fat body in RW medium (%)	41	40	50	55	55	68		54
Retention in fat body in RW + protein (%)			89	89	65	74	85	52

* Experimental injury consisted of multiple punctures, as described in Materials and Methods. The mean haemolymph sugar in the same pupae before injury was 24.9 \pm 1.3 mM.

** Two to four animals were used in each experiment.

*** Numbers of animals used are in parentheses.

Release of trehalose from cecropia fat body in vitro

Pupae in diapause. A series of experiments was carried out with fat body from cecropia pupae in diapause, in which the initial sugar level was generally about double that in the haemolymph (Tables I, II). During incubation in RW medium, up to 60% of the intracellular trehalose was lost, most of it during the first 2 minutes (Fig. 1; Table II, columns A and B). This release of sugar from the tissue was not a consequence of cell lysis, for a maximum of 4% lysis occurred, based upon the release into the medium of glycogen (measured with anthrone after precipitation with ethanol) or upon the previously established retention of capacity for protein synthesis by fat body in a similar medium (Stevenson and Wyatt, 1962). In this and subsequent experiments (see Fig. 2), the rapid initial loss of a certain

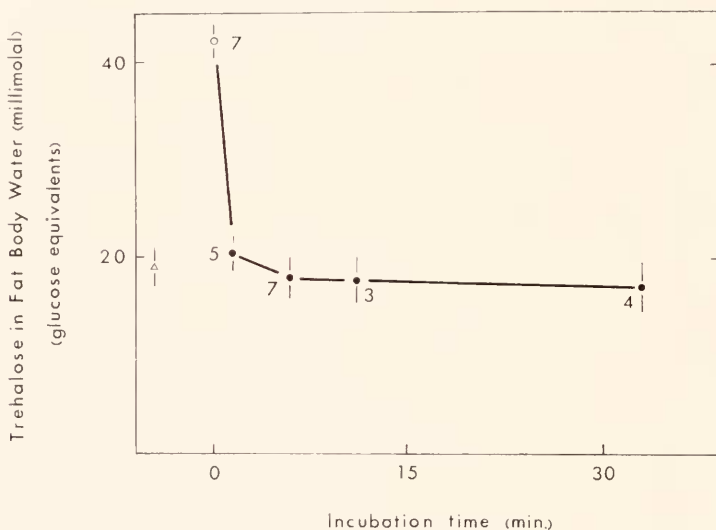


FIGURE 1. Release of trehalose from fat body of diapausing cecropia pupae during 30 minutes incubation in RW medium. Fat body from 4-month-old diapausing pupae (Batch I of Table II) was divided into 4 or 5 portions of 0.1–0.3 g from each pupa, which were separately incubated in 5 ml of RW medium for different times and then taken for analysis; ○, unrinsed fat body; ●, rinsed in RW medium; △, level in hemolymph. Vertical limits are standard errors of the means, and numbers are the numbers of animals used.

fraction of the fat body sugar was followed by little or no further loss during incubation for times up to 30 minutes. The virtual cessation of release could not be due to attainment of diffusion equilibrium, for the volume of medium used was always sufficient to maintain a substantial concentration gradient of sugar toward the exterior (final ratio = 18:1 in the experiment of Fig. 1). These observations are most readily interpreted in terms of intracellular compartmentation: fat body trehalose appears to be divided between rapidly exchangeable and slowly exchangeable compartments.

Since release of trehalose occurred rapidly upon transfer of tissue from its natural milieu of haemolymph to a medium which, while formulated to resemble

haemolymph, lacked protein, we suspected a role of protein in preventing sugar release. We therefore tested the effect of adding cecropia pupal haemolymph protein to RW medium. In the experiment shown in Figure 2, the effects of two levels of protein were tested by incubating tissue for 5 minutes in medium containing 10% protein, which was then diluted with additional medium to 5% and incubation was continued. In the presence of either level of protein, 85–95% of the trehalose in the fat body was retained, compared to only 40% in the protein-free medium. Similar results are shown in Table II (groups C and D).

To determine whether a non-specific protein would also be effective in pre-

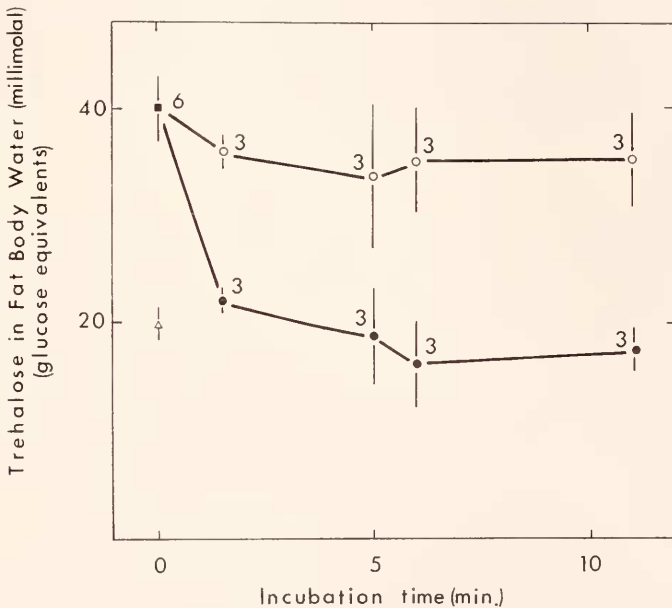


FIGURE 2. The ability of cecropia haemolymph protein to prevent the release of sugar from pupal fat body in RW medium. Experimental details were as in Figure 1, except that one set of fat body samples was placed in RW medium containing 10% pupal haemolymph protein; after 5 minutes, by addition of more medium, the protein was diluted to 5%. The protein was obtained by dialysis of haemolymph from diapausing pupae against 0.01 M phosphate buffer (pH 6.5), followed by lyophilization; ■, unrinsed; ●, RW medium without protein; ○, medium containing protein; △, the level in hemolymph.

venting the release of trehalose from pupal fat body, we tested bovine serum albumin (BSA; Fraction V Powder, Sigma Chemical Corp., St. Louis, Missouri) at several concentrations. The results (Tables II, III) show that, at concentrations above 1%, BSA was as effective as cecropia haemolymph protein. Further evidence for non-specificity of the effect was obtained in experiments with polyvinylpyrrolidone (PVP), a synthetic polymer of approximate M.W. 25,000. PVP was about as effective as BSA, causing (in the experiment of Table II, Column D) the retention of 84% of the initial trehalose, compared with 89% in the presence of BSA. Since it was of further interest to know whether a simple

salt solution containing protein would suffice to maintain trehalose in pupal fat body, a high K^+ - Mg^+ saline containing 2% BSA was also included in this experiment. In this medium, however, trehalose was released to the same extent as in RW medium *without* protein. The predominant difference between RW medium and the saline was the presence of almost 100 mM amino acids in the former, and we infer that the presence of these, together with a macromolecule, is in some way necessary for maintenance of the sugar content of pupal fat body cells. RW medium also contains 8 mM phosphate, which is lacking from the saline, but addition of phosphate to the saline plus BSA did enhance retention of trehalose.

Larvae. In contrast to the situation in pupae, the concentration of trehalose in unrinsed fat body of fifth instar cecropia larvae was somewhat less than that in haemolymph of the same stage (Table I). Further, when larval fat body was incubated for 10 minutes in RW medium either with or without 2% BSA, 50–60% of intracellular trehalose was lost (Table I). Evidently, larval fat body cells were capable of only partial retention of their sugar even in media

TABLE III

Effect of bovine serum albumin (BSA) in preventing release of trehalose from cecropia pupal fat body during incubation in RW medium. Fat body from diapausing pupae (Batch II of Table II) was divided into 3–5 portions per pupa, which were analysed for sugar immediately or after incubation individually for 10 minutes in 5 ml of RW medium containing 0–5% BSA. Sugar concentrations are expressed in molalities as glucose equivalents (mean + S.E.M.)

	Concentration of BSA (%)	No. of Samples	Intracellular sugar (millimolal)	Retention, incubated/unincubated (%)
Unincubated tissue	—	7	38.0 ± 2.8	—
Incubated tissue	0	7	19.0 ± 0.8	50 ± 1
	0.5	3	18.9 ± 1.2	50 ± 2
	1.0	3	32.6 ± 2.8	86 ± 7
	2.0	4	33.9 ± 3.8	89 ± 9
	5.0	3	39.6 ± 0.7	104 ± 1

containing protein, and the tissue acquires the capacity for nearly complete retention during ontogeny in the transition from larva to pupa. In protein-free media, however, similar fractions of intracellular sugar were released from fat body of either stage. These observations indicate significant differences in the relations between cellular and haemolymph trehalose in the two stages.

Effects of temperature on trehalose release from larval and pupal fat body

Since responses to changes in temperature should assist in distinguishing between downhill diffusion and energy-dependent transport as mechanisms for sugar efflux from cells, we examined the release of trehalose from larval and pupal fat body during incubation at different temperatures. In one series of experiments, the progressive appearance of trehalose from fat body in medium at 1°, 25° and 37° was monitored during 10 minutes of incubation (Fig. 3). From pupal tissue, significant trehalose was released at all three temperatures. Although the

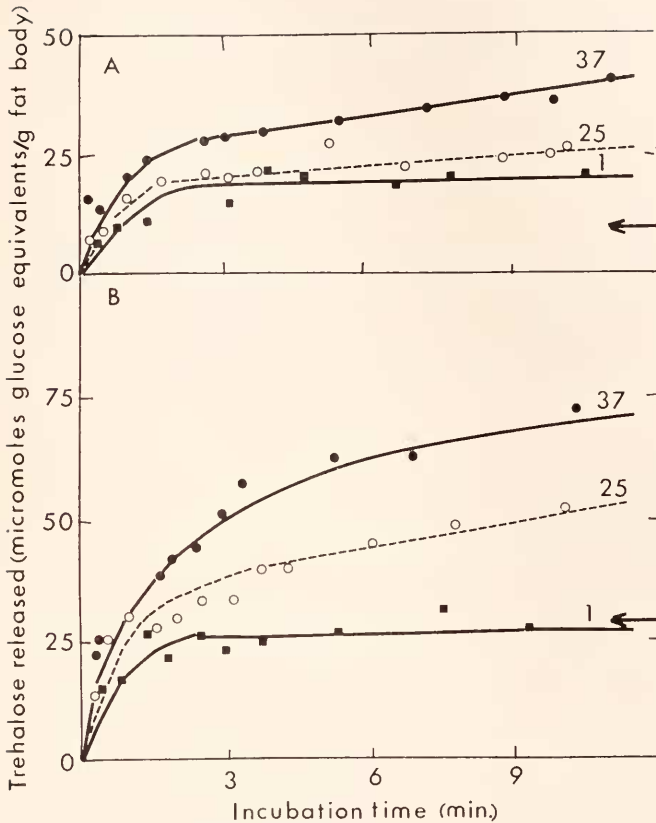


FIGURE 3. Effects of temperature on trehalose release from larval and pupal fat body. Pre-chilled fat body pooled either from (A) diapausing pupae (batch III of Table II) or from (B) feeding fifth instar larvae was divided into three 0.6 g portions, which were incubated in 4 ml of RW medium at 1°, 25° and 37° C, respectively. Samples (5 μ l) of the incubation media were taken for analysis approximately every minute for 10 minutes. Arrows represent the amount of trehalose contributed by haemolymph adhering to the tissue, estimated from the average concentration of trehalose in the haemolymph of the animals and assuming 30% of haemolymph in the blotted tissue samples. Numbers are the temperatures of incubation.

data do not permit calculation of initial rates, it does appear that rates of trehalose efflux were less affected by changes in temperature than would be predicted if they were dependent on an enzyme-catalyzed, energy-dependent process. Our results are consistent with transport by passive diffusion. Larval fat body also released sugar at 25° and 37°, with release at 37° being augmented by net synthesis, as was shown when incubation was prolonged (data not shown). Unexpectedly, when larval tissue was incubated at 1°, the trehalose that appeared in the medium could be fully accounted for by adhering haemolymph. Thus, lowered temperature appeared to prevent sugar efflux from larval, but not from pupal fat body.

In a second series of experiments, the retained trehalose was measured after incubation at five different temperatures for one hour, a period chosen to ensure completion of the initial rapid phase of release (Table IV). Pupal fat body re-

tained an average of 52% of its sugar content, with no significant influence of temperature. Larval tissue, on the other hand, retained only 20% of its sugar at 37° and nearly 80% at 1°, with values for trehalose retained at intermediate temperatures falling between these extremes. The ratios of the quantities of trehalose retained in larval fat body to the quantities retained in pupal fat body decreased with increasing temperature of incubation. The differential effects of incubation temperature on larval and pupal fat body were thus confirmed, the retention of sugar by larval tissue at 1° being particularly notable.

Pharate adults and "transitional" pupae. In early pharate adults at the second or third day of post-pupal development, haemolymph trehalose had risen to 30 mM, well above the level characteristic of pupae (Table I). The level in unripped fat body was approximately equal to that in the haemolymph. Also, about half of the intracellular sugar escaped during 10 minutes in RW medium, whether or not

TABLE IV

Release of trehalose from larval and pupal cecropia silkmoth fat body incubated in RW medium at different temperatures. Larvae reared on foliage and 3 month old unchilled laboratory reared diapausing pupae were acclimated at 27° C. Fat body (0.2-0.4 g) was incubated in 2.0 ml of RW medium for one hour, blotted thoroughly, and intracellular sugar was determined (see Materials and Methods). Results are given in molalities as glucose equivalents, means \pm S.E.M., with numbers of determinations in parentheses. Initial concentrations of trehalose in fat body can be calculated by dividing the concentrations remaining by the decimal fractions remaining. Initial concentrations varied somewhat because the different groups of animals were taken on different days. Haemolymph levels were about 40 mM glucose equivalents for pupae and 100 mM for larvae.

Temperature (°C)	Sugar remaining in fat body after incubation			Retention, remaining sugar/ initial sugar (%)	
	Larval tissue (millimolal)	Pupal tissue (millimolal)	Larval/pupal (ratio)	Larval tissue	Pupal tissue
1	57.9 \pm 7.3 (3)	19.6 \pm 1.0 (4)	3.0	78.7 (3)	55.7 (4)
14	24.0 \pm 2.3 (4)	11.8 \pm 1.3 (5)	2.0	26.8 (4)	50.4 (5)
25	37.5 \pm 2.7 (5)	20.4 \pm 2.9 (6)	1.8	67.3 (5)	48.9 (6)
31	17.5 \pm 2.3 (4)	11.7 \pm 1.3 (5)	1.5	19.3 (4)	52.5 (5)
37	19.1 \pm 2.6 (5)	18.0 \pm 0.6 (4)	0.8	19.8 (5)	51.1 (4)

2% BSA was included. Thus, the relations between fat body and haemolymph sugar in pharate adults resembled those in larvae rather than in diapausing pupae.

Pupae which have been chilled at 6° C for several months are no longer in diapause and have undergone physiological changes preparatory for development, although morphological development characteristic of pharate adults has not yet commenced (Shappirio and Williams, 1957). We termed such animals "transitional pupae." In our experiments, transitional pupae were characterized by fragile fat body tissue, which tended to dissociate into small fragments, and by elevated haemolymph trehalose (above 15 mM) (Table II, group G). We also found that certain groups of pupae which had been stored for several months at 25° C exhibited the characteristics of transitional pupae, even though they had not been chilled and presumably had not undergone endocrine activation for de-

velopment (Williams, 1946); this even included some debrained pupae (Table II, groups E and F). Although their rates of respiration were below $20 \mu\text{l/g-hr}$, all these pupae had haemolymph trehalose at levels well above those characteristic of typical early diapause, and nearly equal to their fat body trehalose. Their fat body also showed diminished capacity to retain intracellular sugar when placed in RW medium plus protein. Thus, in these pupae also, elevated haemolymph sugar was associated with enhanced tendency of the fat body cells to release trehalose.

The transition in the properties of fat body during storage of pupae is also illustrated in experiments on restoration of intracellular sugar after depletion in protein-free medium (Fig. 4). Fat body from pupae stored for 3 months at 25°

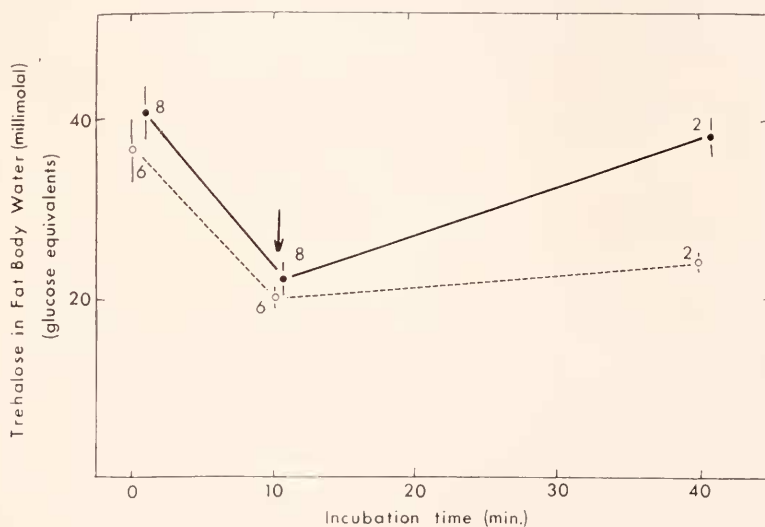


FIGURE 4. The effect of protein in restoring trehalose retentiveness in pupal fat body. Experimental procedure was as in Tables I and II, except that after the tissue was incubated for 10 minutes in protein-free RW medium (arrow), BSA was added to a concentration of 2% and incubation was continued for 30 minutes more; ●, diapause pupae stored 3 months (*cf.* column D of Table II); ○, transitional pupae stored 4.5 months (*cf.* column E of Table II). Vertical limits are standard errors of the means, and numbers are numbers of animals used.

was incubated for 10 minutes in RW medium to allow release of trehalose, whereupon 2% BSA was added. Sugar retentiveness was reestablished and (as a consequence of endogenous trehalose synthesis) its concentration was nearly restored. In a second comparable experiment using pupae that had been stored longer, restoration of intracellular trehalose did not occur, presumably because the cells remained non-retentive upon addition of BSA.

Injured pupae. Another physiological condition of interest is that caused by injury to the integument, a stimulus which, in diapausing pupae, is associated with activation of phosphorylase and breakdown of glycogen in the fat body (Stevenson and Wyatt, 1964), and results in temporarily elevated levels of haemolymph

trehalose (Wyatt, 1961, 1963). In an experiment on this phenomenon, six days after injury, trehalose in both fat body and haemolymph were well above their pre-injury levels (Table II, group H). In addition, tissue placed in medium containing protein retained only 52% of its sugar, which was no more than in medium without protein, and less than that retained by tissue from uninjured diapausing pupae. Hence, the retentiveness of the fat body appeared to be decreased following injury to pupae.

Uptake of trehalose and other solutes into fat body

The experiments on release of trehalose by fat body *in vitro* indicated significant changes during ontogeny of the cecropia silkworm, which suggested that changes in solute uptake might also be found. Penetration into fat body can be measured readily by observing the disappearance of a radioactive solute from

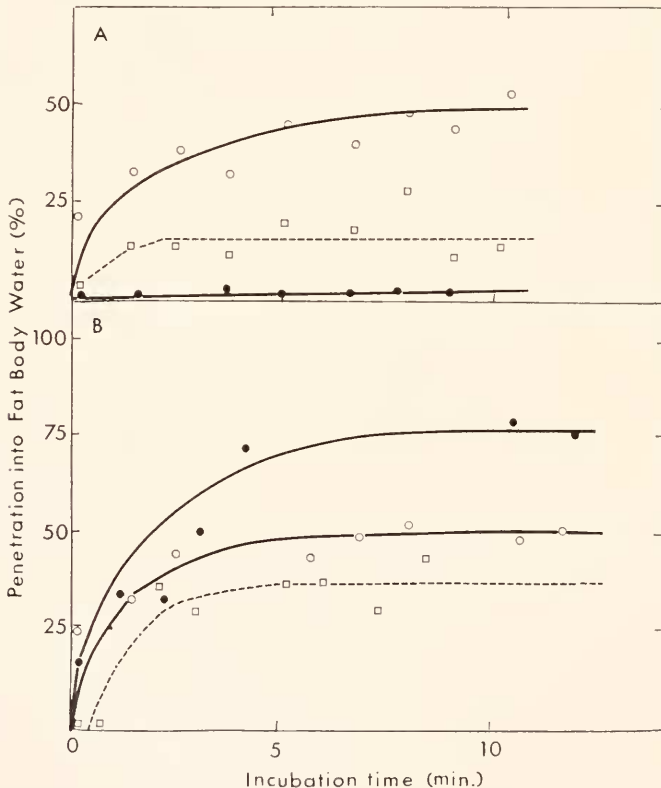


FIGURE 5. Representative curves showing solute penetration into fat body of cecropia silkworm larvae and pupae. Fat body was incubated with ^{14}C -solute together with ^3H -inulin, and percentage penetration into intracellular water was calculated as described in Materials and Methods. (A) Fat body from debrained diapausing pupae. (B) Fat body from fifth stage larvae. Tissue for glycerol and glucose uptake came from larvae 1 and 3 days after spinning; tissue for trehalose uptake was from fully-grown feeding larvae; ●, trehalose; □, glucose; ○, glycerol.

the medium in the presence of a second non-penetrating solute (such as inulin) with a different radioactive label, to serve as a marker for extracellular fluid. If the solute being studied is neither bound nor metabolized appreciably during the course of the experiment, then a measure of its distribution in the intracellular space is obtained.

Penetrability of cecropia fat body. Most experiments were conducted with ^{14}C -trehalose, ^{14}C -glucose or ^{14}C -glycerol, together with ^3H -inulin, as described in Materials and Methods. Typical patterns of uptake into larval and pupal fat body during 10 minutes of incubation are shown in Figure 5. Initial uptake was rapid, but the curves flattened off within 10 minutes at levels well below complete distribution in cell water. This supports the trehalose release data in indicating the

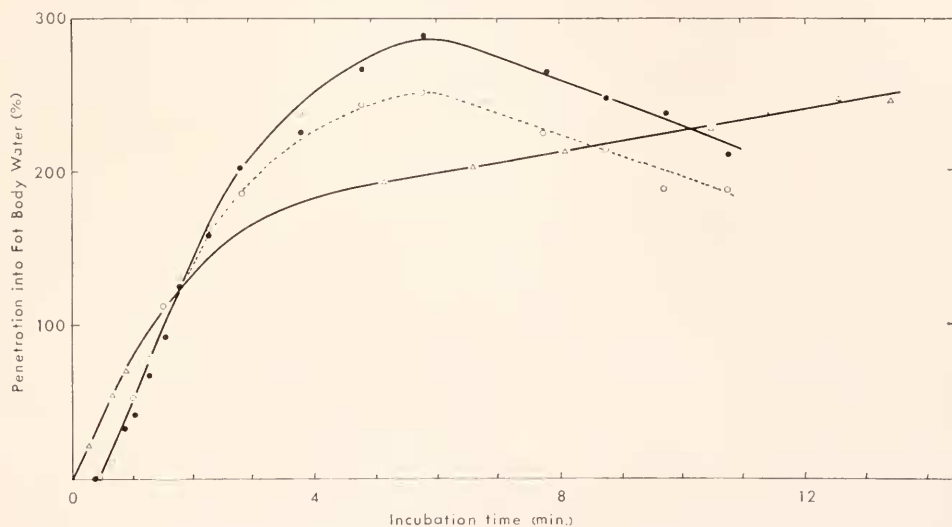


FIGURE 6. Uptake of ^{14}C -glucose *in vitro* into fat body from feeding fifth instar cecropia larvae in the presence or absence of unlabelled glucose or dinitrophenol. Uptake was measured in experiments similar to those of Figure 5. \circ , ^{14}C -glucose, undiluted; \bullet , ^{14}C -glucose plus 50 mM unlabelled glucose. The two foregoing sets of points were obtained with the improved incubation vessel described in Materials and Methods. \triangle , ^{14}C -glucose plus 1.6 mM 2,4-dinitrophenol, preceded by 3 hours of 6×10^{-5} M dinitrophenol *in vivo* (cf. Table VI).

existence of more than one compartment within the tissue. The extent of distribution of trehalose in larval tissue and of all three solutes in pupal tissue did not increase significantly when incubation was extended to one hour; with larval tissue, glucose and glycerol were metabolized during prolonged incubation. That the trehalose which penetrated intracellular water of larval fat body was not bound was shown by rinsing the tissue in $\text{K}^+\text{-Mg}^{++}$ Ringer after incubation with ^{14}C -trehalose and measuring the radioactivity remaining. After two brief rinses, less than 4% of the trehalose that had penetrated the cells was retained.

Evidence on the mechanism of sugar uptake was sought by testing whether the rate of penetration of ^{14}C -glucose was affected by dilution with unlabelled glucose. Glucose, rather than trehalose, was used in this experiment because of its very low

TABLE V

Penetration of trehalose into cecropia fat body in different media. Penetration of ^{14}C -trehalose was measured in experiments similar to those described in Figure 5. Feeding, fifth instar larvae and pupae chilled 5 months were used

Experiment	Source of fat body	Medium and additions	Penetration of trehalose (%)
I	Larvae	RW medium	89
		RW + 50 mM trehalose	81
II	Larvae	RW medium	58
		Pupal haemolymph	80
	Pupae	RW medium	1
		Pupal haemolymph	3

endogenous level (less than 1 mM). Fat body from feeding fifth instar larvae (*i.e.*, before spinning) was used, in which uptake of glucose should be facilitated by its rapid conversion to trehalose. The uptake of ^{14}C -glucose *in vitro* was unaffected by the presence of 50 mM unlabelled glucose, which indicates that the process was not limited by a saturable carrier (Fig. 6). Because of conversion to trehalose, the apparent distribution of glucose in cell water greatly exceeded 100%, and the decline in this value after six minutes of incubation is probably explained by release into the medium of newly-synthesized ^{14}C -trehalose. In the experiments on uptake of ^{14}C -trehalose, we did not attempt to measure initial rates, but the presence of unlabelled trehalose did not alter the 10-minute penetration value (Table V). In the experiments presented in Figure 7 and Tables V-VIII, the medium generally included 50 mM unlabelled solute, and no difference was observed by its presence or absence.

To test whether metabolic energy might be required for maintenance of the fat body's stage-specific characteristics with respect to solute penetration, insects were injected with 2,4-dinitrophenol at doses sufficient to uncouple oxidative phosphorylation (Kurland and Schneiderman, 1959). Time was allowed for dis-

TABLE VI

Penetration of glycerol, glucose and trehalose into fat body of cecropia larvae and pupae treated with 2,4-dinitrophenol. 2,4-Dinitrophenol was injected 1.5 hour before dissection of feeding fifth instar larvae, or 3 hours before dissection of debrained, diapausing pupae, in amounts, estimated to give the stated concentrations when distributed in the haemolymph. In the experiment with larval tissue, dinitrophenol was also included in the incubation medium.

Penetration of solutes into fat body in vitro was measured in experiments similar to those in Figure 5, each using tissue from 3-5 animals

Source of tissue	Dinitrophenol treatment	Penetration into fat body		
		Glycerol (%)	Glucose (%)	Trehalose (%)
Larvae	6×10^{-5} M <i>in vivo</i> , then 1.6×10^{-3} M <i>in vitro</i>	86	244	93
Pupae	4×10^{-5} M <i>in vivo</i>	57	38	19
	2×10^{-4} M <i>in vivo</i>	55	32	6

tribution of the drug, and its effectiveness was observed from the flaccidity of the animals. Fat body was collected and penetration of labelled solutes was measured. The initial rate of glucose uptake into larval fat body treated with dinitrophenol was no different from that in its absence (Fig. 6). In this experiment, the continuing uptake of ^{14}C -glucose after five minutes of incubation may be explained by reduction in trehalose synthesis and stimulation of glycolysis as consequences of uncoupling. From the data in Table VI, it is evident that after dinitrophenol treatment larval fat body took up trehalose freely and pupal fat body did not, just as in its absence (*cf.* Table VII). The penetrations of glucose and glycerol also did not differ significantly from those in untreated larval and pupal tissue, respectively (*cf.* Fig. 7). Thus, neither uptake nor exclusion of sugars or glycerol is dependent upon an unimpaired supply of ATP.

The rapid penetration of trehalose into larval fat body is in marked contrast with the general exclusion of disaccharides by cells of vertebrates. To test whether another disaccharide might also penetrate insect fat body, we used ^{14}C -sucrose. With larval tissue, sucrose distributed in 10 minutes into an apparent 124% of the intracellular water, showing that it penetrated rapidly and was metabolized

TABLE VII

Penetration of disaccharides into fat body. Penetration of ^{14}C -trehalose and ^{14}C -sucrose was measured in experiments similar to those described in Figure 5. Feeding fifth instar larvae and debrained diapausing pupae were used

Source of tissue	Penetration into fat body	
	Trehalose (%)	Sucrose (%)
Larvae	85	124
Pupae (expt. 1)	11	12
Pupae (expt. 2)	12	10

to a small extent (Table VII). Pupal fat body, on the other hand, excluded sucrose to the same extent as it did trehalose.

Changes during development. During development of the cecropia silkworm, no systematic changes were observed in penetration of the fat body by glycerol or glucose, though glycerol generally entered at a faster rate and to a greater extent than did glucose (Figs. 5, 7). Penetration of trehalose, on the other hand, underwent rather striking developmental changes. In fat body from feeding fifth instar larvae, trehalose distributed through 70–90% of the cell water. After the cessation of feeding, penetration of trehalose declined steadily during days 1–5 of prepupal development, approaching zero on the sixth day. During the 48 hours preceding ecdysis, trehalose penetration was restored to 45%, but then it declined in the fresh pupa to remain close to zero throughout diapause. Upon termination of diapause, penetrability to trehalose was restored. In fat body from animals at day 2 of pharate adult development (following activation by chilling) penetration of trehalose had risen to 60%, nearly the level observed with tissue from feeding mature fifth instar larvae. Later stages in pharate adult development

could not be examined because the fat body becomes extremely fragile and cannot be handled.

To get an estimate of how quickly the fat body changes at the termination of diapause, development was initiated in brainless pupae by injection of β -ecdysone. Penetration of trehalose into fat body was still low after 18 hours, but by 42 hours after administration of the hormone it had risen to 53%, a value close to that found for day 2 pharate adults (Table VIII). As in natural development, uptake of glucose and glycerol remained unaltered during the response to ecdysone.

Haemolymph trehalose levels, measured at different stages of development, are also shown in Figure 7, and are consistent with developmental changes reported previously (Bade and Wyatt, 1962; Wyatt, 1967). The correlation of haemolymph trehalose level with penetrability of fat body by trehalose is notable. This sug-

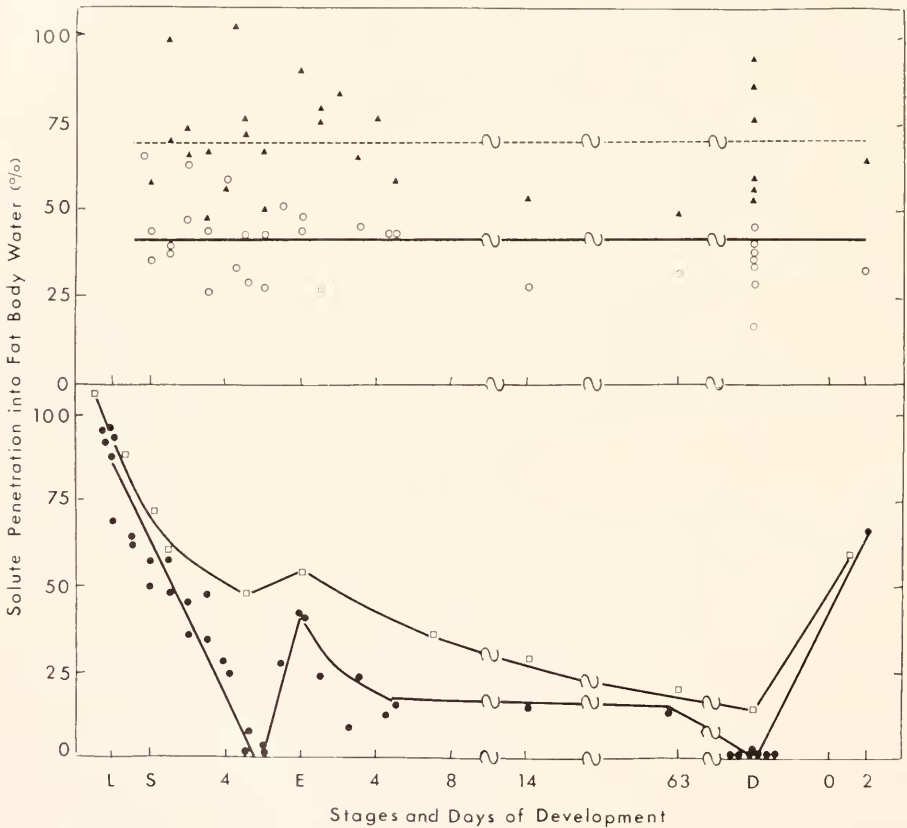


FIGURE 7. Developmental changes in the penetration of solutes into cecropia fat body and in the level of trehalose in the haemolymph; ▲, penetration of glycerol; ○, penetration of glucose; ●, penetration of trehalose; each point is obtained from an experiment similar to those shown in Figure 5; □, level of trehalose in haemolymph (mM, in glucose equivalents) estimated with anthrone in haemolymph pooled from animals at each stage: L, feeding fifth instar larvae; S, day of spinning; E, day of larval-pupal ecdysis; D, diapause. The stage following D is the early pharate adult.

TABLE VIII

Penetration of glycerol, glucose and trehalose into fat body of cecropia pupae after ecdysone injection or injury. Debrained diapausing pupae were selected for respiration less than 20 μ l of O₂ per gram-hour. β -Ecdysone was injected at 2.5 μ g/g pupa weight. Experimental injury was performed as described in Materials and Methods. Penetration of ¹⁴C-solutes was measured in experiments similar to those in Figure 5, each using tissue from 3-5 animals. Sugar levels were estimated with anthrone on pooled haemolymph samples, and are expressed as glucose equivalents

Experiment	Treatment	Penetration into fat body			Sugar level in haemolymph (mM)
		Glycerol (%)	Glucose (%)	Trehalose (%)	
I	β -Ecdysone				
	18 hours	56	40	3	24.3
	42 hours	63	41	5.3	34.2
II	Untreated control	85	42	0	20.2
	Injured				
	2 days	93	44	4	29.2
	5 days	75	16	10	37.6
	9 days	54	31	6	35.6
	20 days				31.4

gests that low blood trehalose is associated with the existence of a barrier between the intracellular water and the haemolymph.

Effects of injury. To test whether the response to injury (see above) was accompanied by altered fat body cell penetrability, eighteen debrained diapausing cecropia pupae were injured and, 2, 5 and 9 days later, fat body was collected and tested *in vitro*. Although the blood sugar level had almost doubled by the fifth day, penetration of trehalose into fat body was found to remain low and unchanged (Table VIII).

Penetrability of fat body in RW medium and in haemolymph. In the experiments that have been described, solute penetration into fat body was measured in RW medium. In case the tissue might behave differently in haemolymph, larval and pupal tissue were assayed for trehalose uptake during incubation in RW medium and in pupal haemolymph (Table V). Larval haemolymph could not be used without dilution because of its rapid clotting. In both media, larval fat body took up trehalose and pupal tissue did not. Therefore, we believe that the uptake of trehalose into fat body in RW medium is a valid indication of the properties of the tissue in its natural milieu of haemolymph.

DISCUSSION

Information on intracellular concentrations of metabolites is essential for understanding the operation *in vivo* of the mechanisms whereby metabolism is regulated. In insects, it is of especial importance to have such data for the fat body, since this tissue, as the chief center for intermediary metabolism and the synthesis of the organic constituents of the haemolymph, must play a key role in homeostasis. Our present data are apparently the first on the sugar content of insect fat body

analyzed without prior rinsing, and they show that even quick rinsing of this tissue in osmotically equivalent salt solutions can lead to loss of 40–60% of the intracellular sugar. Previous data on the sugar content of rinsed fat body (see Introduction) are therefore subject to question. We do not know to what extent a similar caveat applies to other metabolites, but it is unlikely that charged molecules would pass through the cell membrane as readily as sugars.

The present analyses on unrinsed tissue, corrected for adhering haemolymph, indicate sugar (chiefly trehalose) in fat body water at levels that range from somewhat less than to more than double those in the surrounding haemolymph. Sugar levels in fat body below those in haemolymph raise a question concerning how the cells can supply sugar to the blood without active transport, a process for which no evidence exists in this system. The calculated intracellular concentrations are subject to some error because of uncertainty in estimating both the water content of the fat body and the amount of adhering haemolymph, properties which must vary with species and stage of development. If the actual water content is less than that assumed (70% or 50% of cell weight according to stage and species), the true intracellular concentrations would be higher than those calculated. That the mean sugar concentration in fat body water may actually be lower than that in the blood, however, is possible in view of indications of intracellular compartmentation which will be discussed below. One compartment might contain sugar at a relatively low level, while the level in another was high enough to permit transfer to the haemolymph by diffusion.

Intracellular sugar levels substantially above those in the haemolymph were observed in adult *Blaberus* and in diapausing pupae of the cecropia silkworm. In these stages, the ability of the tissue to supply trehalose to the haemolymph is apparent, but a question arises as to how higher-than-ambient sugar levels are maintained in the cells. Upon brief incubation in an isosmotic medium containing inorganic ions and amino acids in proportions approximating those in lepidopteran haemolymph, sugar was lost from cecropia pupal tissue to the same extent as it was from larval and pharate adult fat body. Tissue from pupae in diapause, however, was distinctive in that its sugar content was largely retained when protein was included in the medium. This presumably accounts for its ability to maintain intracellular sugar above the haemolymph level *in vivo*. In our experiments, 2% bovine serum albumin or polyvinylpyrrolidone could substitute for cecropia haemolymph protein. But in the absence of amino acids, in a simple inorganic Ringer solution designed for lepidopteran tissue, fat body failed to retain its sugar even when protein was present. Thus, some synergism is implied between a macromolecule (protein or PVP) and amino acids in "sealing" the fat body cell membranes. In fat body from non-diapause stages, protein had little effect in preventing loss of intracellular sugar. On the other hand, when escape of trehalose from fat body was measured at different temperatures, larval tissue retained its intracellular sugar at 1° C while pupal tissue did not. This may reflect an adaptive change in membrane function at pupation, for pupae normally encounter below-freezing temperatures in nature. Thus, these two properties of fat body, namely retention of trehalose in protein-containing medium and temperature dependence of trehalose release, both change in the transition from larva to pupa of the cecropia silkworm.

The implied developmental changes in cecropia fat body were confirmed and more fully explored by measurements of the penetration of radioactive solutes. In contrast to penetrability of glucose or glycerol, which did not change significantly during ontogeny, trehalose penetrated freely during the larval and pharate adult stages but scarcely during the pupal diapause. Measurements at daily intervals during the prepupal period showed additional transient changes. Unlike *H. cecropia*, diapause or non-diapause *M. sexta* failed to exhibit such changes during ontogeny (data not shown). In contrast to the changes in sugar release, those in the capacity of fat body to take in trehalose were manifest in protein-free medium. This is difficult to understand, and appears to indicate some asymmetry in the control of membrane transport. The developmental changes in cecropia fat body function with respect to both efflux and uptake of trehalose presumably reflect alterations in membrane structure.

The mechanism of sugar uptake by cecropia fat body cells has not been studied in detail, but the available evidence does not support the existence either of active transport or of a substrate-specific, carrier-mediated process like that known in mammalian tissues, including adipose tissue (Wilbrandt and Rosenberg, 1961; Crofford and Renold, 1965). Uncoupling of oxidative phosphorylation with dinitrophenol had no effect on penetration. Uptake of ^{14}C -glucose or trehalose was not affected by the presence of a high level of the non-radioactive compound. Sucrose, like trehalose, readily entered larval, but not pupal, fat body. These observations are consistent with simple diffusion, and suggest that transport into and out of fat body may be restricted by sieving based on molecular size, the pupal tissue admitting monosaccharides but not disaccharides.

The experiments on sugar release and those on uptake by fat body both gave results indicative of some sort of compartmentation within this tissue. The initial rapid release (within 2 minutes) of 40–60% of the intracellular trehalose was followed by little further loss during 30 minutes of additional incubation, even though a concentration gradient from cell contents to medium was always maintained. In other preliminary experiments (data not given), fat body transferred to fresh medium after 1 hour of incubation in RW medium, released little additional sugar. In our experiments on uptake, initial rapid transfer was followed by relative stability. The extent of occupation of cell water during 10 minutes incubation averaged 65% for glycerol and 40% for glucose, but ranged from zero up to 95% for trehalose depending on the stage of development. By comparison, in rat heart muscle, it has been stated that only 75% of the intracellular water was available for sugar distribution (Morgan, Henderson, Regen and Park, 1961). While there is kinetic evidence for functional biochemical compartmentation within various cell types (Threlfall and Heath, 1968; Sols and Marco, 1970), it is difficult to conceive of appropriate physical compartmentation in fat cells to account for our observations. Possibly, the nucleus or other organelles might constitute a compartment which does not readily exchange its trehalose with that in the haemolymph. Alternatively, although the fat body appears histologically homogeneous, some of its cells might exchange sugars less readily than others. Further study of this phenomenon is indicated.

The level of trehalose in the haemolymph of an insect at any time is a function of the rates of synthesis and utilization. Periods during which metabolic ac-

tivity is high should be associated with increased use of trehalose, which might be expected to lower the level in the haemolymph. In cecropia, metabolic rates can be inferred from measurements of oxygen consumption (Schneiderman and Williams, 1953). This is maximal in the mature larva, which is also characterized by high haemolymph trehalose, however, and minimal during the pupal diapause, when haemolymph trehalose is low. Hence, the haemolymph trehalose level must be regulated primarily by the rate of production, not that of utilization. The fat body is the chief site of trehalose synthesis, and the output from this tissue may be influenced by (a) the supply of precursors, (b) the activities of enzymes and (c) release from the cells to the haemolymph. Let us consider each of these interdependent factors in turn.

Trehalose may be synthesized from free glucose after phosphorylation by hexokinase, from glycogen via the action of phosphorylase, or from glucose phosphates produced by gluconeogenesis. The transient rise in blood non-reducing sugar after glucose was fed to silkworms (Kuwana, 1937) is an example of stimulation of trehalose synthesis from the first source. The rise in blood trehalose after injury to cecropia pupae must be due, at least in part, to phosphorolytic breakdown of glycogen (Stevenson and Wyatt, 1964). But the elevation of trehalose after either of these stimuli is followed by return to "normal" levels, suggesting the resumption of control by long-term homeostatic mechanisms. As a possible regulatory mechanism at the level of precursor supply, Friedman (1968, 1971) has pointed to the activation of *Phormia* glucose-6-phosphatase by trehalose, which might lower the availability of glucose-6-phosphate for trehalose synthesis.

The rate-limiting enzyme in trehalose synthesis in cecropia fat body is trehalose-6-phosphate synthetase, which is subject to inhibition by trehalose (Murphy and Wyatt, 1965). Such feedback inhibition is, in our view, the principal mechanism operating to stabilize the level of this disaccharide in the fat body (cf. Friedman, 1967). Trehalose levels within the cecropia fat body vary much less than those in the haemolymph, and are regularly about half as high in pupal as in larval tissue. The mechanism by which trehalose is maintained at a lower level in the pupal than in the larval fat body is unknown, but, in this connection, the possibility that altered intracellular Mg^{++} may modulate the activity of the synthetase *in vivo* is currently being explored (Jungreis and Wyatt, 1972).

Our findings on the changing penetrability of fat body to trehalose provide an explanation for the markedly lower haemolymph sugar in the cecropia pupa than in the larva. In the larva, where trehalose escapes freely from the cells, its level must build up throughout the blood space before its intracellular synthesis can be blocked by feedback. In the diapausing pupa, since trehalose is retained in the fat body, intracellular feedback occurs while the level in the haemolymph remains far below that in the tissue.

The observations on certain intermediate developmental stages strengthen the case for an important role of fat cell penetrability in regulating hemolymph trehalose. Just before the larval-pupal ecdysis, a transient rise in penetrability to trehalose is accompanied by a simultaneous rise in blood sugar. In early pharate adults (obtained either by incubation of chilled pupae or by injection of ecdysone) increased penetrability and elevated haemolymph sugar appeared simultaneously. Certain batches of pupae which had been stored for more than 4 months (although

still in diapause by the criterion of respiration) showed elevated blood sugar correlated with decreased capacity of fat body to retain intracellular trehalose. Fat body from injured pupae showed both elevated internal sugar (presumably resulting from activation of phosphorylase) and a decrease in ability to retain it (even though the low penetrability to external trehalose was unaltered), the two effects presumably contributing to bring about the rise in haemolymph sugar that is observed after injury.

The properties of insect fat body with respect to transport of sugars and the regulation of haemolymph sugar thus merit further attention, being profoundly different from the analogous functions of the tissues of vertebrates.

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SUMMARY

1. The levels of sugar (chiefly trehalose) have been determined in haemolymph and in unrinsed fat body (corrected for adhering haemolymph) of developing stages of the silkmoth *Hyalophora cecropia*, as well as a few individuals of other insect species. In cecropia, the fat body trehalose level was relatively stable at about 45 millimolal in mature larvae and about 20 millimolal in pupae, while that in the haemolymph ranged from somewhat above the tissue level in larvae to less than half the tissue level in diapause pupae.

2. During brief incubation in culture medium, fat body released 40–60% of its trehalose content. In larval tissue only, this release was sensitive to temperature, being blocked at 1° C. In fat body from diapausing pupae only, it was blocked by the presence of 1% or more of haemolymph protein, bovine serum albumin or polyvinylpyrrolidone in a medium containing amino acids.

3. Penetration of ¹⁴C-solutes into fat body cells was measured. Trehalose and sucrose penetrated readily into larval fat body but not into pupal fat body; penetrability was restored in the early pharate adult. Glucose and glycerol distributed in 40–65% of cell water at all stages of development. Uptake was unaffected by dinitrophenol or by dilution with unlabelled solute. The data suggest that the mechanism of transport is simple diffusion restricted by molecular size.

4. The partial release of internal sugar during incubation of fat body and the partial occupation of intracellular water by exogenous solutes indicate the existence of some form of compartmentation in the fat body.

5. The developmental changes in retentiveness and penetrability to trehalose in cecropia fat body may explain changes in the steady state level of haemolymph sugar. In the larva, feedback inhibition of intracellular trehalose synthesis does not take place until trehalose has built up throughout the haemolymph, whereas in the diapausing pupa, the fat body retains trehalose, and feedback can occur within the cells while haemolymph trehalose remains low.

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